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Award Number: W81XWH-10-1-0176

TITLE: Suppression of BRCA2 by Mutant Mitochondrial DNA in Prostate Cancer

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REPORT DATE: February 2012

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. REPORT DATE | 2. REPORT TYPE | 3. DATES COVERED |
|---|---|---|
| 01-02-2012 | Annual | 1 FEB 2011 - 31 JAN 2012 |

**4. TITLE AND SUBTITLE**
Suppression of BRCA2 by Mutant Mitochondrial DNA in Prostate Cancer

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**
W81XWH-10-1-0176

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**
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**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
University of Texas Southwestern Medical Center

Dallas, TX 75390

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**
Mutations in mitochondrial DNA (mtDNA) are frequent in prostate cancer and they seem to occur early during prostate malignant transformation. Depletion of mtDNA in prostate cancer cells has been linked to acquisition of androgen-independence, progression to an invasive phenotype that is resistant to conventional chemotherapies, as well as induction of epithelial-mesenchymal transition leading to cancer metastasis. Using long-range genomic polymerase chain reaction, large deletion of mtDNA can be detected in prostate cancer tissues but not benign or normal prostate tissues. Noticeably, our study excludes the germ-line origin of the mutant mtDNA pattern in prostate cancer patient through analysis of the blood of the corresponding patient. Our data conclude that mtDNA deletion is due to carcinogenesis process in somatic prostate cells.
In addition, our data have unveiled the molecular alteration in prostate cancer cells resulted from mtDNA deletion. For example, Skp2 protein elevation is often associated in prostate cells with loss of mtDNA. Also, the presence of Skp2 expression can decrease the expression of BRCA2 protein as an early biomarker of prostate neoplastic transformation, which is due to BRCA2 proteolysis.

**15. SUBJECT TERMS**
No subject terms provided.

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | 19b. TELEPHONE NUMBER *(include area code)* |
| U | U | U | UU | 14 | USAMRMC |

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## INTRODUCTION

Mitochondrial DNA (mtDNA) depletion has been shown to promote malignant progression of prostate cancer cells. However, the molecular mechanisms underlying the association between mutant mtDNA and prostate cancer progression remain obscure. Mutant mtDNA has been associated with increased genomic DNA double-strand breaks. The resulting genomic instability could account for the multiple phenotypic effects observed in prostate cells harboring mutations/depletion of mtDNA, i.e. increased migration, acquisition of androgen-independence and progression to an invasive phenotype that is resistant to conventional chemotherapies. *BRCA2* is among the few genes known to be involved in repair of DNA double-strand breaks, and its loss confers a significantly elevated risk to develop aggressive, rapidly progressing, high-grade prostate carcinoma. During the first year we have identified the presence of large mtDNA deletions in prostate cancer specimens but not in age-matched benign prostate hyperplasia, and we have correlated their presence with loss of BRCA2 protein in cancer, overall suggesting that BRCA2 loss might be an important molecular determinant of prostate cancer progression induced by mutant mtDNA.

## BODY

During the second year we have accomplished most of the planned experimental tasks. Part of the experiments planned for the second year has been already performed during the first year, as detailed below.

**Aim 1. Investigate the association between mutations in mtDNA and loss of BRCA2 protein in prostate cancer specimens *in vivo* (Months 1-19)**

*Task 2. Measure the activity of mitochondrial respiratory complexes in human prostate cancer specimens (Months 9-15)*

2.b Correlate mtDNA mutations with changes in mitochondrial complexes' activity (*Months 13-15*)

An inverse correlation (Spearman's correlation coefficient: - 0.67) was found between activity of mtDNA respiratory complexes, which was significantly decreased in prostate cancer, and the number of mtDNA deletions (Table 1).

*Task 3. Measure BRCA2 protein expression in human prostate cancer specimens (130 samples) (Months 12-19)*

3.a Prepare total protein lysates from low-grade vs. high-grade PCA frozen biopsies and analyze BRCA2 protein levels by immunoblotting *(Months 12-18)*

3.b Analyze BRCA2 protein expression in paraffin-embedded prostate specimens by immunohistochemistry *(Months 12-18)*

3.c Investigate the correlation between loss of BRCA2, mtDNA mutations, tumor stage *(Months 18-19)*

Tasks 3.a and 3.b have been completed during this first year, and the results have been published [1].

Task 3.c. We prepared protein lysates from 6 BPH and 20 PCA (10 high-grade and 10 low-grade PCA) and analyzed the correlation between loss of BRCA2 protein and presence/number of mtDNA large deletions in PCA. We found a correlation between loss of BRCA2, number of mtDNA mutations and Gleason grade (Table 2; Spearman's correlation coefficient: -0.89 for loss of BRCA2 and number of mtDNA deletions; + 0.76 for number of mtDNA deletions and Gleason grade).

**Aim 2. Identify the molecular mechanisms of down-regulation of BRCA2 expression by mutant mtDNA (Months 10-24)**

*Task 1. Investigate the regulation of BRCA2 expression by mutant mtDNA at the transcription, translational, post-translational level (Months 10-19)*

1.b Determine BRCA2 mRNA levels in C4-2 *versus* LNCaP cells, in wild-type LNCaP *versus* LNCaP Rho-, in PNT1A/C4-2 cybrids versus PNT1A wild-type by RT-PCR (*Month 13*)

We have analyzed BRCA2 mRNA levels and found no difference in mtDNA-mutated cells (Figure 1).

1.c Determine BRCA2 protein translation rate in wild-type and mutant mtDNA cells by incorporation of a radiolabeled aminoacidic precursor, followed by immunoprecipitation with anti-BRCA2 antibody and SDS-PAGE analysis (*Months 14-16*)

We measured BRCA2 protein translation and found a significant decrease in the rate of translation of BRCA2 in mtDNA-mutated cells (Figure 1). We extended our analysis to other types of mtDNA-depleted/mutated cancer cells.

1.d Determine BRCA2 protein half-life in wild-type and mtDNA-mutated prostate cells by pulse-chase experiments. Eventually, identify proteolytic pathways involved in BRCA2 protein degradation, using specific inhibitors, and determine involvement of the ubiquitin ligase Skp2 in BRCA2 ubiquitination and degradation by Skp2 siRNA followed by quantification of total and ubiquitinated BRCA2 protein levels by immunoblotting (*Months 17-19*)

We determined BRCA2 protein half-life by incubating the cells with cycloheximide and measuring BRCA2 protein levels by Western blotting. We found a significant decrease in BRCA2 protein half-life in all mtDNA-mutated cells tested (Figure 2). We also analyzed the expression of Skp2, an E3 ubiquitin ligase involved in ubiquitination and proteasomal degradation of BRCA2 [2], and of miR-1245, a miRNA recently shown to target BRCA2 3'-UTR and to inhibit its translation [3]. Skp2 protein and miR-1245 were increased in mtDNA-depleted cells (Figure 3 and data not shown). Inhibition of Skp2 expression by siRNA or use of a miR-1245 inhibitor alone partially recovered BRCA2 levels but their combination was able to completely restore BRCA2 to wild-type levels (Figure 3).

*Task 2. Identify proteins differentially regulated by mutant mtDNA that may regulate loss of BRCA2 expression (Months 19-24)*

2.a Identify proteins differentially regulated by mutant mtDNA using Clontech antibody microarrays and confirm results by immunoblotting (*Months 19-21*)
2.b Determine the role of differentially-expressed molecules in regulating BRCA2 loss using gain/loss of-function genetic approaches and/or pharmacological inhibition (*Months 21-24*)

We are in the process of performing these experiments.

**Aim 3. Investigate the role of BRCA2 in preventing/hindering mtDNA-related prostate cancer progression (Months 19-36)**
*Task 1. Generate clones of C4-2, LNCaP Rho-, PNT1A/C4-2 cybrids and PNT1A Rho0 cells stably overexpressing BRCA2 (Months 19-21)*
1.a Stably transfect mtDNA mutant cells with BRCA2 cDNA or empty vector. Eventually, first subclone BRCA2 cDNA in an inducible system of expression *(Months 19-21)*

We were able to generate C4-2 cells overexpressing BRCA2, but they show a mitotic arrest and it is impossible to perform experiments. Thus we have subcloned BRCA2 cDNA in a doxycycline-regulated system (pTet-OFF from Clontech) and generated stable clones overexpressing BRCA2 upon removal of doxycycline.

*Task 2. Assess the role of BRCA2 in preventing tumor growth and metastasis in pre-clinical animal models (Months 21-32)*
2.a To assess the role of BRCA2 in preventing tumor growth, inject athymic nude mice (Balb/c) subcutaneously with mock- and BRCA2-expressing mtDNA-mutant cells [C4-2 and LNCaP Rho-, 2 injecting sites *per* mouse, 10 animals/cell type; mock LNCaP and mock-"mtDNA reverted" LNCaP used as control, 2 injecting sites *per* mouse, 10 animals/cell type], measure tumor growth, harvest tumors for histological examination and immunohistochemical analysis of BRCA2 expression. Total: 60 mice requested. (*Months 21-30*)

We plan to start this task in the next months.

2.b Generate stable clones expressing luciferase (*Months 22-23*)
We tried to generate stable mtDNA-mutated clones overexpressing luciferase without success. We now think that it would be unlikely the generation of these clones because luciferase uses ATP for its activity, thus it depletes mtDNA-mutated cells of the energy required for anabolic processes and survival. Of note, mtDNA-mutated cells have reduced ATP levels due to dysfunctional oxidative phosphorylation.

**KEY RESEARCH ACCOMPLISHMENTS**
- Demonstration of correlation between loss of BRCA2 and number of mtDNA deletions in PCa.
- Demonstration of reduced translation and protein stability of BRCA2 in mtDNA-mutated cells

- Demonstration of inverse correlation between loss of BRCA2 protein and increase in Skp2 levels and miR-1245 in mtDNA-mutated cells

## REPORTABLE OUTCOMES

### Publication

## CONCLUSION

Our results provide evidence for a translational and post-translational mechanism of regulation of loss of BRCA2 protein following mtDNA mutations, that implicates both Skp2 and miR-1245 upregulation. In addition, we provide evidence for an inverse correlation between BRCA2 protein levels, the number of mtDNA deletions and Gleason grade in prostate carcinoma, suggesting that accumulation of mtDNA deletions may occur with progression of prostate cancer and further decrease BRCA2 levels in PCa.

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[2]L. Moro, A.A. Arbini, E. Marra, M. Greco, Up-regulation of Skp2 after prostate cancer cell adhesion to basement membranes results in BRCA2 degradation and cell proliferation. J Biol Chem 281 (2006) 22100-22107.

[3]L. Song, T. Dai, Y. Xie, C. Wang, C. Lin, Z. Wu, Z. Ying, J. Wu, M. Li, J. Li, Up-regulation of miR-1245 by c-myc targets BRCA2 and impairs DNA repair. J Mol Cell Biol 4 (2012) 108-117.

## APPENDICES

**SUPPORTING DATA**

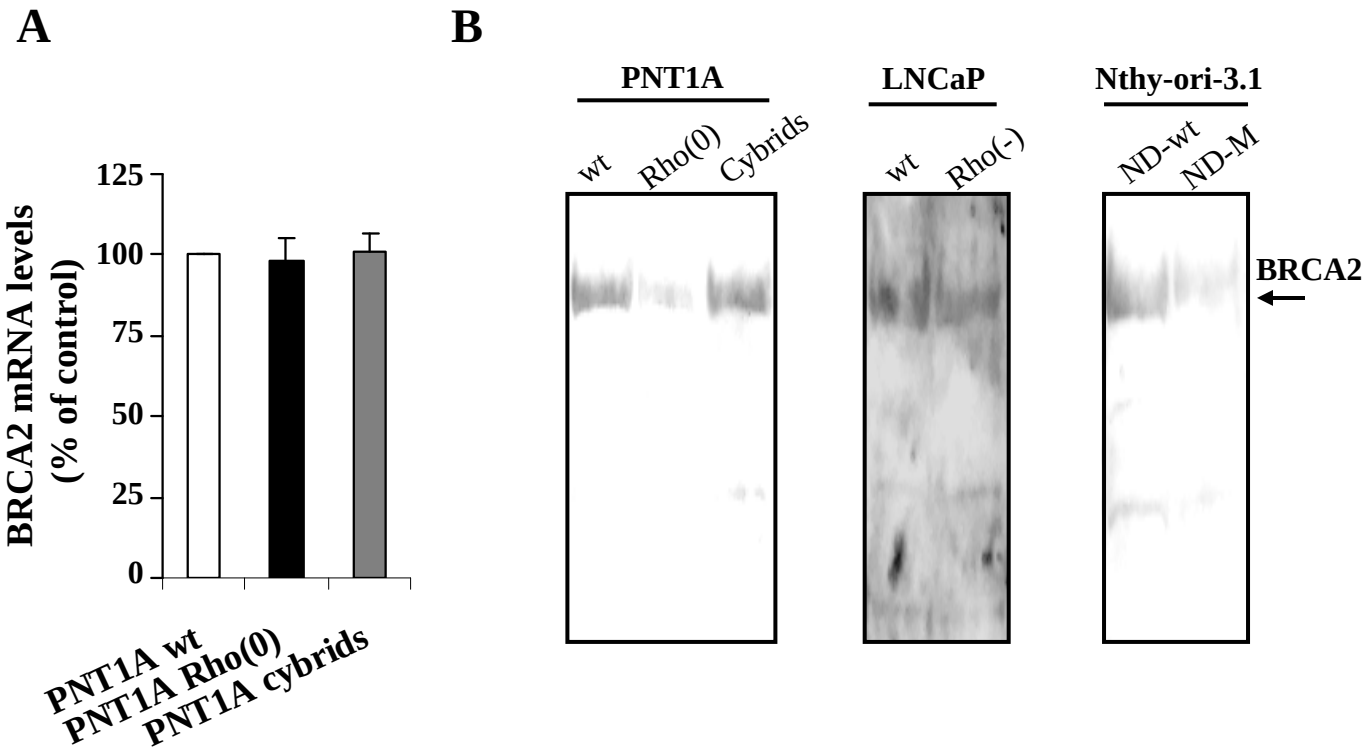
**Tables 1-2**
**Figures 1-3**

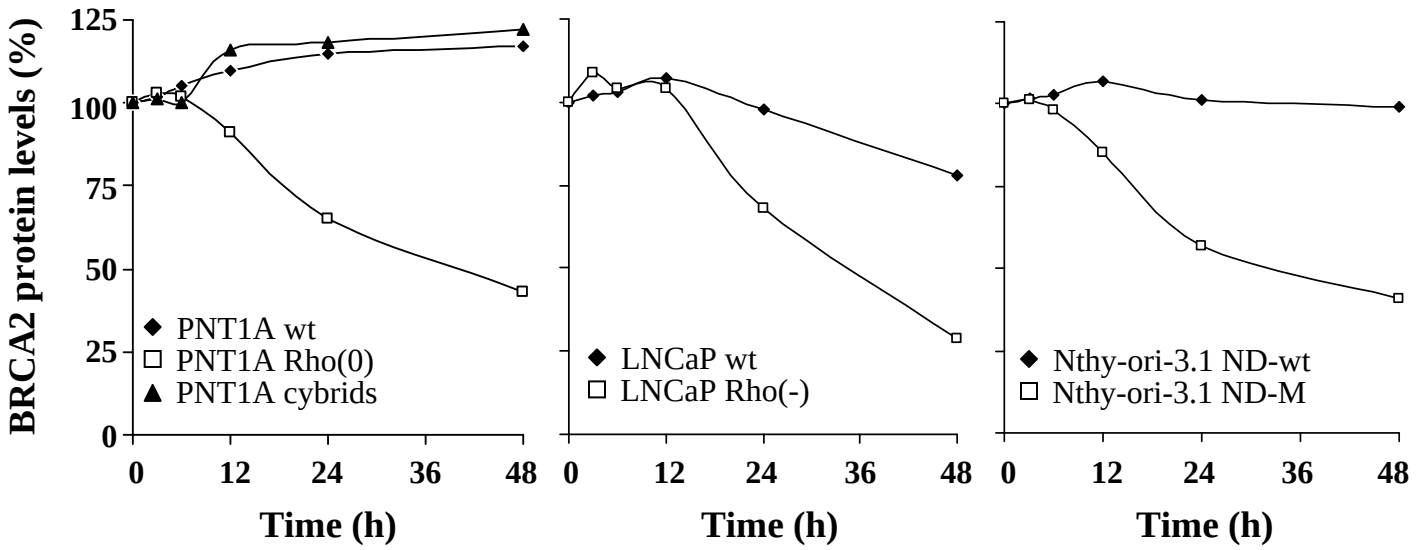
**Figure legends**

**Fig. 1 BRCA2 protein translation is reduced in mtDNA-mutated cells. A**, BRCA2 mRNA levels were quantified in PNT1A wild-type, Rho(0) and cybrids cells by real-time RT-PCR and expressed as percentage of wild-type cells. **B**, Wild-type and mtDNA-mutant cells were metabolically-labeled with $^{35}$Smethionine/cysteine for 3 h, and proteins were immunoprecipitated with an antibody to BRCA2. Immunoprecipitated proteins were electrophoresed on 4-8% Tris-acetate NuPage gels and $^{35}$S-labeled proteins were visualized by fluorography.

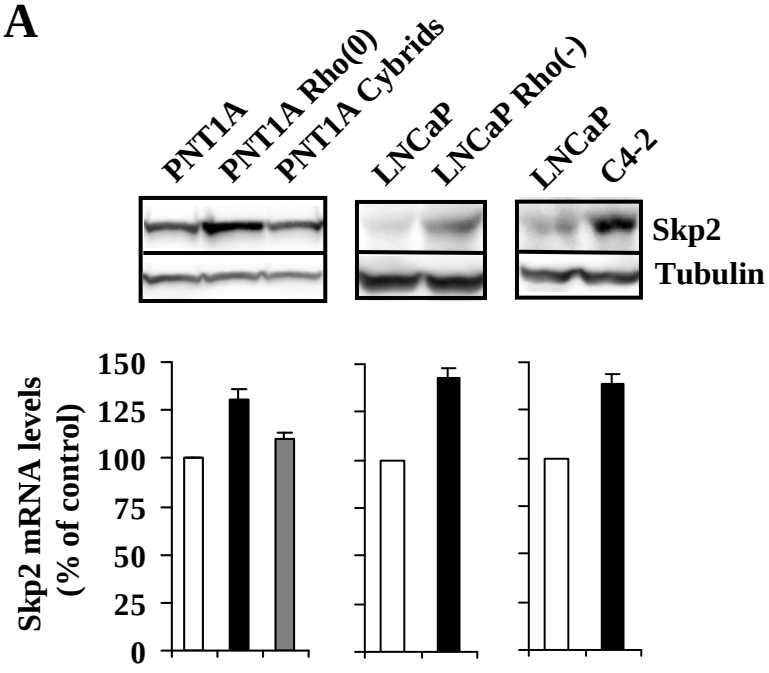
**Fig. 2 BRCA2 protein stability is reduced in mtDNA-mutated cells.** The half-life of BRCA2 protein was evaluated by addition of cycloheximide (20 mg/ml) to wild-type and mtDNA-mutant cells: BRCA2 steady-state protein levels were determined by Western blotting analysis up to 48 h after addition of cycloheximide and reported in the graphs as percentage of the BRCA2 protein levels at time 0.

**Fig. 3 MtDNA mutations increase Skp2 protein and miR-1245 levels. A**, Skp2 protein and mRNA levels were analyzed in wild-type and mtDNA mutant cells by Western blotting and real-time RT-PCR, respectively. **B**, Wild-type and mtDNA-mutant cells were transiently transfected with Skp2 siRNA or non-specific siRNA and, after 48 h, analyzed for Skp2 and BRCA2 protein levels by Western blotting. **C**, Wild-type and mtDNA-mutant cells were transiently transfected with a miR-1245 inhibitor and/or Skp2 siRNA or non-specific siRNA. Forty-eight h after transfection, BRCA2 levels were analyzed by Western blotting.

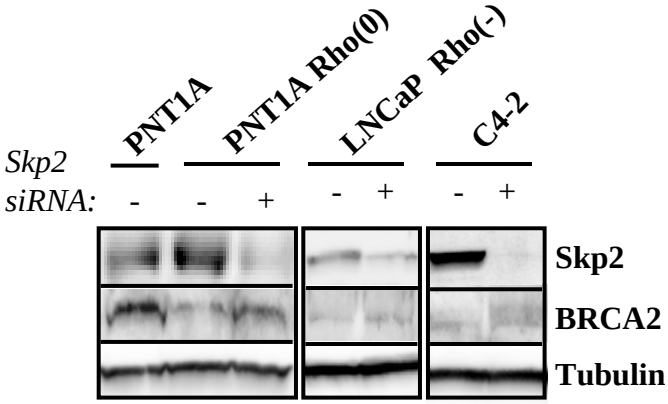

A
B
BRCA2 mRNA levels
(% of control)
125
100
75
50
25
0
PNT1A wt
PNT1A Rho(0)
PNT1A cybrids
PNT1A
LNCaP
Nthy-ori-3.1
wt
Rho(0)
Cybrids
wt
Rho(-)
ND-wt
ND-M
BRCA2


BRCA2 protein levels (%)
125
100
75
50
25
0
0
12
24
36
48
Time (h)
PNT1A wt
PNT1A Rho(0)
PNT1A cybrids
LNCaP wt
LNCaP Rho(-)
Nthy-ori-3.1 ND-wt
Nthy-ori-3.1 ND-M

**A**


PNT1A
PNT1A Rho(0)
PNT1A Cybrids
LNCaP
LNCaP Rho(-)
LNCaP
C4-2
Skp2
Tubulin
Skp2 mRNA levels (% of control)
150
125
100
75
50
25
0

**B**


PNT1A
PNT1A Rho(0)
LNCaP
Rho(-)
C4-2
Skp2 siRNA:
- - + - + - +
Skp2
BRCA2
Tubulin

**C**

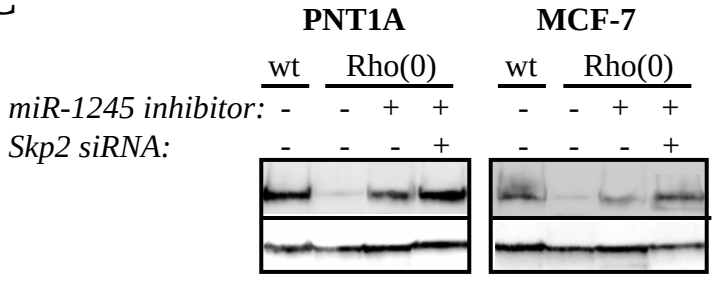

PNT1A
MCF-7
wt
Rho(0)
wt
Rho(0)
miR-1245 inhibitor: - - + + - - + +
Skp2 siRNA: - - - + - - - +

**Table 1. Activity of mitochondrial respiratory complexes and mtDNA deletions**

| **Prostate Tissues** | **Complex I/III** *(nmol/min/mg protein)* | **Complex II/III** *(nmol/min/mg protein)* | **Complex IV** *(nmol/min/mg protein)* | **N. of mtDNA deletions** |
|---|---|---|---|---|
| **BPH (1-6)** | 52 ± 4 | 21 ± 4 | 11 ± 3 | 0.5 |
| **PCa 1** | 26 ± 4 | 16 ± 3 | 6 ± 2 | 16 |
| **PCa 2** | 39 ± 3 | 21 ± 2 | 9 ± 2 | 5 |
| **PCa 3** | 41 ± 2 | 18 ± 4 | 9 ± 1 | 6 |
| **PCa 4** | 21 ± 4 | 12 ± 3 | 7 ± 3 | 13 |
| **PCa 5** | 19 ± 3 | 16 ± 3 | 6 ± 3 | 16 |
| **PCa 6** | 21 ± 2 | 18 ± 1 | 8 ± 1 | 13 |
| **PCa 7** | 23 ± 4 | 9 ± 3 | 9 ± 3 | 10 |
| **PCa 8** | 29 ± 3 | 11 ± 2 | 6 ± 2 | 8 |
| **PCa 9** | 31 ± 2 | 17 ± 2 | 7 ± 1 | 6 |
| **PCa 10** | 41 ± 5 | 18 ± 3 | 10 ± 2 | 4 |
| **PCa 11** | 39 ± 1 | 21 ± 3 | 8 ± 2 | 3 |
| **PCa 12** | 42 ± 4 | 18 ± 4 | 5 ± 1 | 3 |
| **PCa 13** | 21 ± 4 | 11 ± 3 | 7 ± 3 | 7 |
| **PCa 14** | 19 ± 1 | 20 ± 3 | 6 ± 3 | 11 |
| **PCa 15** | 14 ± 2 | 18 ± 1 | 8 ± 1 | 14 |
| **PCa 16** | 17 ± 4 | 16 ± 3 | 5 ± 1 | 10 |
| **PCa 17** | 19 ± 3 | 11 ± 2 | 6 ± 2 | 7 |
| **PCa 18** | 35 ± 5 | 17 ± 4 | 7 ± 1 | 4 |
| **PCa 19** | 50 ± 4 | 22 ± 1 | 10 ± 2 | 0 |
| **PCa 20** | 36 ± 3 | 17 ± 2 | 7 ± 1 | 3 |

**Table 2. Loss of BRCA2 expression and mtDNA deletions in prostate cancer**

| Patient No. | Age (y) | Gleason Grade | mtDNA Deletions (No.) | BRCA2 Levels (arbitrary units) |
|---|---|---|---|---|
| 1350 | 55 | BPH | 0 | 0.4, 0.4 |
| 1391 | 61 | BPH | 0 | 0.5, 0.5 |
| 1465 | 67 | BPH | 0 | 0.8, 0.8 |
| 1519 | 58 | BPH | 1 | 0.7, 0.7 |
| 1526 | 61 | BPH | 2 | 0.9, 0.8 |
| 1365 | 60 | BPH | 2 | 0.4-0.5 |
| 1116 | 59 | PCa (4) | 4 | 0.07, 0.07 |
| 1150 | 66 | PCa (7) | 3 | 0.3, 0.4 |
| 1153 | 56 | PCa (7) | 0 | 0.3, 0.4 |
| 1208 | 69 | PCa (7) | 5 | 0.1, 0.1 |
| 1248 | 65 | PCa (7) | 4 | 0.2, 0.1 |
| 1285 | 70 | PCa (7) | 5 | 0.1-0.2 |
| 1251 | 86 | PCa (7) | 3 | 0.25-0.2 |
| 2208 | 62 | PCa (7) | 6 | 0.1-0.1 |
| 2160 | 62 | PCa (7) | 5 | 0.2-0.25 |
| 2178 | 60 | PCa (7) | 5 | 0.2-0.2 |
| 2238 | 59 | PCa (7) | 5 | 0.3-0.25 |
| 2114 | 58 | PCa (7) | 6 | 0.3-0.25 |
| 1147 | 66 | PCa (8) | 16 | 0.1-0.05 |
| 1838 | 74 | PCa (8) | 8 | 0.01-0.02 |
| 1211 | 66 | PCa (9) | 3 | 0.15-0.2 |
| 1266 | 63 | PCa (9) | 5 | 0.3-0.4 |
| 1314 | 64 | PCa (9) | 4 | 0.4-0.3 |
| 2167 | 76 | PCa (9) | 7 | 0.1-0.1 |
| 2164 | 54 | PCa (9) | 13 | 0.1-0.2 |
| 1727 | 61 | PCa (9) | 10 | 0.01-0.02 |
| 1898B | 60 | PCa (9) | 11 | 0.01-0.02 |
| 2371 | 65 | PCa (9) | 13 | 0.01-0.02 |
| 2472 | 53 | PCa (9) | 14 | 0.01-0.02 |
| 2096 | 63 | PCa (9) | 16 | 0.01-0.01 |

BPH, **benign prostatic hyperplasia**
PCa, **prostate carcinoma**